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(54) **Title:** ANTIOXIDANT COMPOSITION FOR TISSUE AND ORGAN PRESERVATION

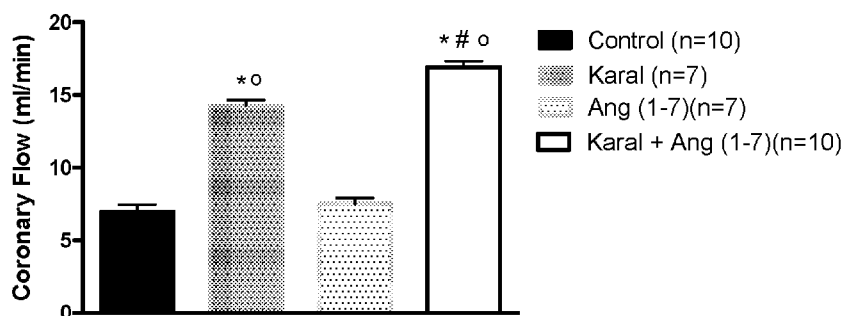


Figure 7

(57) **Abstract:** The present invention relates to an antioxidant composition, comprising a combination of at least one carbonic acid selected from the group of mono- and polycarbonic acids, at least one mono- or polysaccharides derivatized heterolytic aldehyde, at least one selenium-compound such as seleniommethionine, seleniuncysteine, or selenite, at least one compound selected from the group of sulfur-, disulfide- or sulfhydryl-containing amino acids such as N-acetyl- L-methionine and N-acetyl-L-cysteine, and amino-sulfonic acids such as taurine, at least one carbohydrate, and at least one Mas receptor agonist such as angiotensines. The invention further relates to a pharmaceutical composition comprising such an antioxidant composition and a pharmaceutically acceptable carrier. The invention is particularly useful in a solution for preserving organs and tissue, particularly during the process of transplantation.



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ANTIOXIDANT COMPOSITION FOR TISSUE AND ORGAN PRESERVATION

The present invention relates to a new antioxidant solution able to preserve the cardiac function during warm (coronary occlusion) and prolonged cold (cardioplegy and 6 hour storage) ischemia.

Ischemia/ reperfusion injury plays an important role in the cardiological as well the surgical field. (stenting, myocardial infarction, vascular as well as cardiac surgery.) Furthermore it plays a major role in the field of transplantation on post transplant organ function and patients' survival.

Heart transplantation (HTX) has emerged as the most effective therapy for end- stage heart failure. Because of the increasing divergence between the availability and the demand of organs, special interest has to be drawn to optimize the long-term success of each transplant. Various risk factors, antigen- dependent as well as antigen -independent, have been identified to be responsible for initial and late graft performance, thus influencing patients' post transplant life quality and expectancy. (Large SR. Is there a crisis in cardiac transplantation? The Lancet, 2002; 359 (9308):803-804; Wilhelm MJ, Pratschke J, Laskowski I, Tilney NL. Ischemia and reperfusion injury Transplantation Reviews, 2003;17 (3): 140-157).

The most significant antigen-independent insults that transplanted organs experience are transient episodes of warm ischemia related to donor hypotension, the process of brain death or revascularization, as well as an extended period of cold ischemia during organ preservation and storage, followed by ischemia-reperfusion (I/R) injury. (Wilhelm MJ, Pratschke J, Laskowski I, Tilney NL. Ischemia and reperfusion injury Transplantation Reviews, 2003;17 (3): 140-157; Vassalli G, Gallino A, Weis M, von Scheidt W, Kappenberger L, von Segesser LK, Goy JJ. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. European Heart Journal, 2003; 24 (13): 1180-1188; Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM Jr. Heart preservation for transplantation: Principles and Strategies. Annals of Thoracic Surgery, 1999; 68: 1983-1987).

One of the underlying mechanisms of myocardial I/R injury is the generation of reactive oxygen and nitrogen species (RONS). Peroxynitrite (ONOO-) formed by the reaction of superoxide anion (O₂⁻) and nitric oxide (NO[•]) remains a potent contributing factor to I/R injury. (Zweier JL, Hassan Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovascular Research* 2006;70:181-190). RONS do not only cause acute events like stunning, infarction or potentially lethal arrhythmias; they are also present in the chronically failing heart. (Giodarno FJ. Oxygen, oxidative stress, hypoxia and heart failure. *Journal of Clinical Investigation* 2005;115(3):500-508). High levels of lipid peroxidation, found in routine follow-up myocardial biopsies, indicate a persistence of oxidative stress contributing to a chronic rejection known as graft coronary artery disease (GCAD), which still remains the leading cause of death within the first year after HTX (Taylor DO, Edwards LB, Aurora P, Christie JD, Dobbels F, Kirk R, Rahmel AO, Kucheryavaya AY, Hertz MI. Registry of the International Society for Heart and Lung Transplantation: 25th Official Adult Heart Transplant Report-2008. *The Journal of Heart and Lung Transplantation*, 2008; 27 (9): 943-956; Schimke I, Schikora M, Meyer R, Duebel HP, Modersohn D, Kleber FX, Baumann G. Oxidative stress in the human heart is associated with changes in the antioxidative defense as shown after heart transplantation. *Molecular and Cellular Biochemistry*, 2000; 204: 89-96).

Cardiac dysfunction correlates with an imbalance between RONS and NO (ONOO-/NO ratio). The reduced availability of NO and the increase of the formation of ONOO- leads to vascular dysfunction (Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circulation Research* 2000; 87: 840-844). In the heart, NO₂⁻ has the potential to act as an endogenous store of NO especially liberated during ischemia, when the generation of NO from L-arginine by NOS enzymes is impaired. On the other hand, an inactivation of ONOO- also prevents the nitration on amino acids like L-arginine forming nitro-L-arginine known as a potential inhibitor of NOS activity formed easily during ischemia/reperfusion.

Modulating the generation of RONS, either by enhancing the endogenous defense mechanisms, or by administering substances with free radical scavenging abilities showed beneficial effects on the cardiovascular system, not only in experimental (Supinski GS, Callahan LA. Polyethylene- Glycol Superoxid dismutase prevents endotoxin-induced cardiac dysfunction. *American Journal of Respiratory and Critical Care Medicine* 2006;173:1240-1247; Gandhi C, Upaganalawar A, Balaraman R. Protection against *in vivo* focal myocardial ische-

mia/reperfusion injury induced arrhythmias and apoptosis by hesperidin. *Free Radical Research* 2009;43(9):817-827), but also in clinical settings, particularly in patients affected by an increased free radical load (Saliba W, El Fakih R, Shaheen W. Heart failure secondary to selenium deficiency, reversible after supplementation. *International Journal of Cardiology* 2008, doi: 10.1016/j.ijcard.2008.11.095; Odermarsky M, Lykkesfeldt J, Liuba P. Poor vitamin C status is associated with increased media thickness, decreased microvascular function, and delayed myocardial repolarization in young patients with type 1 diabetes. *American Journal of Clinical Nutrition* 2009;90:447-452).

It was recently reported that oxidative stress plays an important role within the surgical stress response leading to an impairment of the NO regulating system. Especially during transplantations, where prolonged episodes of I/R are present, the reduction of eNOS activity is aggravated. Modulation of oxidative stress by administration of antioxidants has been shown to reduce morbidity and mortality in various patient populations (Humphrey CD, Pittman FE. A simple methylene blue-azure II basic fuchsin stain for epoxi- embedded tissue sections. *Stain Technology* 1974; 42:9-14).

Karal® is a new antioxidant infusion solution consisting of α -ketoglutaric acid (α KG), 5 hydroxy-methyl-furfural (5 HMF), trace amounts of N-Acetyl-Selenium-L-Methionine and N-Acetyl-L-Methionine.

α KG, an intermediate of the citric cycle, is located in the cytosol and the inner mitochondrial membrane and important for several mechanisms including (i) the transamination of NH_4^+ , (ii) the oxidative decarboxylation to succinyl-CoA, CO_2 and $\text{NADH}+\text{H}^+$ which itself transfer its reduction equivalent to ubiquinon necessary for ATP production, (iii) the gluconeogenesis of lactate and (iv) scavenging hydrogen peroxide (H_2O_2). It is assumed, that α KG also may interact with peroxynitrite (ONOO^-) (Halliwell B. and Gutteridge J. *Free Radicals in Biology and Medicine, Third Edition, 1999, Oxford Press*). Nitrated or oxidized forms of arginine by ONOO^- are able to inhibit NO^+ production by blocking NOS. Furthermore, a direct modification of NO^- synthase (NOS) by ONOO^- results in an overproduction of superoxide anion radicals ($\text{O}_2^{\cdot-}$) which itself reacts with NO^+ forming ONOO^- (Sun J, Druhan LJ, Zweier JL. Dose-dependent Effects of Reactive Oxygen and Nitrogen Species on the Function of Neuronal Nitric Oxide Synthase. *Arch Biochem Biophys.* 2008; 471:126-33).

It was shown, that the myocardial content of α KG decreases during cardiac surgeries and that by providing this substance added to blood cardioplegia during coronary artery bypass grafting, metabolic abnormalities could be reduced and thus cardiac protection achieved. (Kjellmann U, Bjoerk K, Ekroth R, Karlsson H, Jagenburg R, Nilsson F, Svensson G, Wernerman J. α -ketoglutarate for myocardial protection in heart surgery. *The Lancet* 1995;345:552-55).

5-HMF was recently reported as an in vitro acting antioxidant which results in a reduced protein oxidation by free radicals (e.g. H_2O_2) or RONS (e.g. ONOO⁻). Furthermore, 5-HMF inhibited myeloperoxidase activity and increased the expression of glutathione enzymes and superoxide dismutase (Li YX, Yong L, Zhong-Ji Q, Moon-Moo K, Se-Kwon K. In Vitro Antioxidant Activity of 5-HMF Isolated from Marine Red Alga *Laurencia undulate* in Free Radical Mediated Oxidative Systems. *J. Microbiol Biotechnol.* 2009;19(11):1319-1327).

Adequate intake (plasma level of 70-90 μ g/l) of the essential trace mineral selenium is required for optimal activity of endogenous antioxidant enzymes like glutathione- peroxidases or thioredoxin- reductases. Enhanced oxidative stress due to selenium -deficiency has been correlated to heart failure. (Saliba W, El Fakih R, Shaheen W. Heart failure secondary to selenium deficiency, reversible after supplementation. *International Journal of Cardiology*, 2008, doi: 10.1016/j.ijcard.2008.11.095; Navas Acien A, Bleys J, Guallar E. Selenium Intake and Cardiovascular Risk: What is New? *Current Opinion in Lipidology*, 2008; (19):43-49).

Another, already well described cardioprotective substance, is the endogenous peptide Angiotensin (1-7) [Ang. (1-7)], an active biological product of the renin- angiotensin system (Santos RA, Ferreira AJ, Pinheiro SV, Sampaio WO, Touyz R, Campagnole-Santos MJ. Expert Opinion on Investigational Drugs, 2005; 14: 1019-1031). Several experimental studies showed its antiarrhythmic, ameliorating post-ischemic contractile function, antiproliferative and vasodilative effects (Ferreira AJ, Santos RAS, Almeida AP. Angiotensin (1-7): Cardioprotective effect in myocardial ischemia/reperfusion. *Hypertension*, 2001; 38 (2): 665-668; Ferreira AJ, Santos RAS, Almeida AP. Angiotensin (1-7) improves the post-ischemic function in isolated perfused rat hearts. *Brazilian Journal of Medical and Biological Research*, 2002; 35: 1083-1090; Tallant EA, Diz DI, Ferrario CM. Antiproliferative actions of angiotensin (1-7) in vascular smooth muscle. *Hypertension*, 1999; 34: 950-957; Sampaio WO, Santos RAS, Faria-Silva R, Machado LT, Schiffrin EL, Touyz RM. Angiotensin-(1-7) through receptor Mas

mediates endothelial Nitric Oxide synthase activation via Akt-dependent pathways. Hypertension, 2007 49: 185-192).

Ang. (1-7) has been tested in clinical studies and shown to be effective and safe within the human being-at least until the maximum daily dose of 100 µg/ kg (Ueda S, Maemoto SM, Wada A, Ishii M, Brosnihan KB, Umemura S. Angiotensin-(1-7) potentiates bradykinin-induced vasodilatation in man. Journal of Hypertension, 2001; 19(11):2001-2009; Sasaki S, Higashi Y, Nakagawa K, Matsuura H, Kajiyama G, Oshima T. Effects of angiotensin-(1-7) on forearm circulation in normotensive subjects and patients with essential hypertension. Hypertension, 2001; 38 (1):90-94; Rodgers KE, Oliver J, di Zerega GS. Phase I/II dose escalation study of angiotensin 1-7 [A(1-7)] administered before and after chemotherapy in patients with newly diagnosed breast cancer. Cancer Chemotherapy and Pharmacology, 2006; 57:559-568; Rodgers KE, Xiong S, di Zerega GS. Effect of angiotensin II and angiotensin (1-7) on hematopoietic recovery after intravenous chemotherapy. Cancer Chemotherapy and Pharmacology, 2003; 51: 97-106; Soto- Pantoja DR, Menon J, Gallagher PE, Tallant EA. Angiotensin (1-7) inhibits tumor angiogenesis in human lung cancer xenografts with a reduction in vascular endothelial growth factor. Molecular Cancer Therapeutics 2009 Jun; 8(6):1676-1683).

It is an object of the present invention to provide an antioxidant composition/solution useful in preserving organs and tissues, particularly during the process of transplantation.

It is another object of the present invention to provide an antioxidant composition/solution useful in the treatment of conditions and diseases associated with oxidative and nitro-stress.

This object is achieved by an antioxidant composition comprising a combination of

- at least one carbonic acid selected from the group of mono- and polycarbonic acids,
- at least one mono- or polysaccharides derivatized heterolytic aldehyde,
- at least one selenium-compound such as seleniummethionine, seleniumcysteine or selenite,
- at least one compound selected from the group of sulfur-, disulfide- or sulfhydryl-containing amino acids such as N-acetyl-L-methionine and N-acetyl-L-cysteine, and aminosulfonic acids such as taurine,
- at least one carbohydrate, and
- at least one Mas receptor agonist such as angiotensines.

The term “at least one” as used herein means that either one or more than one of each of the aforementioned components may be present in the composition.

In one embodiment only one of each of the aforementioned components is present in the composition, and, accordingly, the antioxidant composition comprises a combination of

- a carbonic acid selected from the group of mono- and polycarbonic acids,
- a mono- or polysaccharides derivatized heterolytic aldehyde,
- a selenium-compound such as seleniummethionine, seleniumcysteine or selenite,
- at least one compound selected from the group of sulfur-, disulfide- or sulfhydryl-containing amino acids such as N-acetyl-L-methionine and N-acetyl-L-cysteine, and aminosulfonic acids such as taurine,
- a carbohydrate, and
- a Mas receptor agonist such as angiotensines.

In one embodiment the carbonic acid is selected from the group of α -ketoglutarate, isocitrate, or a pharmaceutically acceptable salt thereof.

In one embodiment the mono- or polysaccharides derivatized heterolytic aldehyde is selected from the group of 5-hydroxy-methylfurfural, dehydroascorbic acid, maltol, vanillin and furan-2-aldehyde.

In one embodiment the selenium-compound is selected from the group of seleniummethionine, seleniumcysteine and selenite.

In one embodiment the Mas receptor agonist is selected from the group consisting of angiotensines such as angiotensin (1-7) and angiotensin (1-7) analogues.

In one embodiment the carbohydrate is selected from the group consisting of monosaccharides, disaccharides, oligosaccharides and polysaccharides. In one embodiment the carbohydrate is glucose or fructose.

In one embodiment the antioxidant composition comprises a combination of α -ketoglutarate, 5-hydroxy-methylfurfural, N-acetyl-selenium-L-methionine, N-acetyl-L-methionine, glucose and angiotensin (1-7).

In one specific embodiment the antioxidant composition comprises a combination of
0.01 to 500 g/L α -ketoglutarate,
0.001 to 100 g/L 5-hydroxy-methylfurfural,
1.4 to 2.3 mg/L N-acetyl-selenium-L-methionine,
70 to 230 mg/L N-acetyl-L-methionine,
0.1 to 800 g/L mM glucose, and
0.22 ng/L to 2.2 mg/L angiotensin (1-7).

Accordingly, a further feature of the invention is a pharmaceutical composition comprising the antioxidant composition as defined above and a pharmaceutically acceptable carrier.

A "pharmaceutically acceptable carrier" in the meaning of the present invention can be any substance used for the preparation of pharmaceutical dosage forms and known to those skilled in the art. The antioxidant composition and the pharmaceutical composition according to the invention, respectively, preferably are in the form of an aqueous solution.

In one embodiment the antioxidant composition or the pharmaceutical composition according to the invention is for treatment of conditions and diseases associated with oxidative and nitro-stress. Representative examples for conditions and diseases associated with oxidative and nitro-stress are e.g. conditions and diseases associated with ischaemia/reperfusion, cancer as well as hormonal disorders/imbances relating to thyroid gland, hypothalamus and pituitary gland.

In another embodiment the antioxidant composition or the pharmaceutical composition according to the invention is used in a solution for preserving organs and tissues.

In yet another embodiment the antioxidant composition or the pharmaceutical composition according to the invention is used in the preparation of a perfusion solution for preserving organs and tissues during the process of transplantation.

In one embodiment the antioxidant composition or the pharmaceutical composition according to the invention is for treatment of cardiovascular diseases, preferably conditions and diseases associated with ischaemia/reperfusion.

A further feature of the invention is a graft treated with an antioxidant composition or with a pharmaceutical composition according to the invention as defined above and in the claims. The term “graft” as used herein refers to biological material derived from a donor, wherein the biological material is intended to be transplanted into a recipient. Both donor and recipient are preferably mammalian, most preferably human. In one embodiment the graft is selected from the group consisting of tissue, isolated cells and an organ. The organ is preferably a heart.

The chemical properties inside and outside of the tissues and organs are stabilized by the antioxidant composition/solution according to the invention and/or reversed if pathophysiological incidents took place (e.g. before occlusion).

The present invention further relates to a method comprising pre-treating a graft before transplantation with an antioxidant composition or with a pharmaceutical composition according to the invention as defined above and in the claims.

The present invention evaluates, for the first time, the effects of α -ketoglutarate (AKG) and 5-hydroxy-methylfurfural (5-HMF) on peptides containing aromatic amino acids and arginine (e.g. Ang 1-7). This protection prevents the hydroxylation and nitration of these peptides through free radicals and RONS (e.g. peroxynitrite) during reperfusion after warm and prolonged cold ischemia and thus saves their function, preserving organs and tissues during transplantation. Moreover, the invention evaluates for the first time the mechanism and the efficacy of the above-mentioned antioxidant solution Karal® and Ang. (1-7) on cardiac function and myocardial protection in different experimental settings linked to the process of organ transplantation (see accompanying examples and figures below). Angiotensin (1-7) shows vasodilative, anti-proliferative and anti-arrhythmogenic properties. These properties are reduced during ischemia/reperfusion. α -ketoglutarate and 5-HMF protect on one hand the nitration and therefore the function of angiotensin (1-7; specially on tyrosine and arginine) and prohibit the inactivation of NOS.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1- (A) The effect of 5, 10 and 20% Karal®, on the incidence and duration of reperfusion arrhythmias (ASI = arrhythmia severity index). (B) Percentage of irreversible arrhythmias

in hearts perfused with KRS containing 5, 10 and 20% Karal®. (C) The effect of Karal® 5% and Ang (1-7) 0.22 nmol/L on the incidence and duration of reperfusion arrhythmias. (D) Percentage of irreversible arrhythmias in hearts perfused with KRS containing Karal® 5% and Ang (1-7) 0.22 nmol/L.

Figure 2– (A,B) The effect of 5, 10 and 20% Karal®, on the coronary flow during basal, occlusion and reperfusion time. **(C,D)** The effect of Karal® 5% and Ang (1-7) 0.22 nmol/L on coronary flow during basal, occlusion and reperfusion time.

Figure 3– The effect of 5, 10 and 20% Karal®, on the systolic **(A)** and diastolic tension **(B)** during and after occlusion. The effect of Karal® 5% and Ang (1-7) 0.22 nmol/L on the systolic **(C)** and diastolic tension **(D)** during and after occlusion.

Figure 4– The effect of 5, 10 and 20% Karal®, on the nitrite concentration.

Figure 5– Determination of peroxynitrite (ONOO-) scavenging function of Karal® with chemiluminescence technique.

Figure 6– Determination of peroxynitrite (ONOO-) scavenging function of Karal® and Ang (1-7) with chemiluminescence technique.

Figure 7– Coronary flow in cold ischemia using Karal 20% alone or in combination with Ang. (1-7).

Figure 8– Systolic tension in cold ischemia using Karal 20% alone or in combination with Ang. (1-7).

Figure 9– Scanning electron microscopy of the endothelial sheet of rat heart endocardium. **(A)** Native rat heart, freshly excised, no cardioplegy, no reperfusion. **(B)** Rat heart endothelial cells perfused with Karal 20% plus Angiotensin (1-7). **(C)** Rat heart endothelial cells perfused with Karal 20%. **(D)** Rat heart endothelial cells perfused with Angiotensin (1-7). **(E)** Rat heart endothelial cells perfused with Krebs Ringer solution (control).

Figure 10– Electron micrographs. **(A)** Native rat heart, freshly excised, no cardioplegy, no reperfusion. **(B)** Rat heart endothelial cells perfused with Karal 20% plus Angiotensin (1-7).

(C) Rat heart endothelial cells perfused with Karal 20%. (D) Rat heart endothelial cells perfused with Angiotensin (1-7). (E) Rat heart endothelial cells perfused with Krebs Ringer solution (control).

DETAILED DESCRIPTION OF THE TECHNOLOGY

In the present technology we evaluate the effectiveness of the combination of Karal® (Table 1) with Ang (1-7) on organs and tissues preservation during the process of organ transplantation.

To test the effects of this combination the experiments were performed perfusing the hearts either with KRS plus Ang. (1-7) 0.22nmol/L alone (n=5) or in combination with Karal® 5% (n=5) after stabilization. The concentration of Ang. (1-7) was chosen as it was proven to be effective in isolated hearts in previous studies (Ferreira AJ, Santos RAS, Almeida AP. Angiotensin (1-7): Cardioprotective effect in myocardial ischemia/reperfusion. Hypertension, 2001; 38 (2): 665-668; Ferreira AJ, Santos RAS, Almeida AP. Angiotensin (1-7) improves the post-ischemic function in isolated perfused rat hearts. Brazilian Journal of Medical and Biological Research, 2002; 35: 1083-1090).

Table 1 – Composition of Karal® solution

COMPOUND	AMOUNT	CONCENTRATION (mmol)
α -ketoglutarate	9.0 g	61.64
5-hydroxy-methylfurfural	3.0 g	23.79
N- Acetyl-Selenium-L-methionine	2.0 mg	0.0084
N-Acetyl-L -methionine	100.0mg	0.52
Glucose	30.0 g	166.52
H ₂ O	1 litre	

The present invention can be better understood by the following examples, which are illustrated herein for the purpose of the description and should not be regarded as limiting:

EXAMPLE 1: Preparation and perfusion of isolated hearts (Warm Ischemia)

Male Wistar rats, 12-14 weeks old, 250-300 g, were decapitated 10 to 15 minutes after intraperitoneal injection of 400 IU of heparin (ROCHE, Brazil). Thoracotomy was performed and the heart was rapidly separated from its neighboring structures and excised. Through a 1.0 ± 0.3 cm aortic stump the heart was attached to the Langendorff apparatus and perfused with Krebs's Ringer Solution (KRS). The perfusion fluid was maintained at $37 \pm 1^\circ\text{C}$, with a pressure of 70 mmHg and constant oxygenation (5% CO_2 /95% O_2). A force transducer (model TSD 104 A, Biopac) was attached through a heart clip to the apex of the ventricles to record the contractile force (tension, g) on a computer, by a data-acquisition system (Biopac System, Santa Barbara, CA). A diastolic tension of $1.0 \pm 0.2\text{g}$ was applied to the hearts. Electrical activity was recorded with an ECG (Nihon Kohden, Japan) with the aid of 2 cotton wicks placed directly on the surface of the right atrium and left ventricle (bipolar lead). Coronary flow was measured by collecting the perfusate over a period of 1 minute at regular intervals. The hearts were perfused for an initial 20-minute period with KRS. After this period, the hearts were perfused for an additional 20-minute period with KRS (control, $n=5$) or with KRS containing Karal® solution (compounds – Table 1) in the concentrations of 5%; 10% or 20% (minimum of $n=5$).

After the equilibration period, the left anterior descending coronary artery (LAD) was ligated according to the method described by Lubbe (Lubbe WF, Daries PS, Opiel LH. Ventricular arrhythmias associated with coronary antifibrillatory action of antiarrhythmic agents. Cardiovascular Research, 1978; 12: 212-220), beneath the left auricular appendage together with the adjacent veins. The ligature was released after 15 minutes, and reperfusion with different KRS (above) was performed for additional 30 minutes. Cardiac arrhythmias were defined as the presence of ventricular tachycardia and/or ventricular fibrillation after the ligature of the coronary artery was released. The incidence and duration of the post-ischemic arrhythmias were observed in each experiment and classified according to the arrhythmia severity index (ASI) (Bernauer W, Ernenputsch I. Antagonistic effects of alpha -adrenoceptor blocking agents on arrhythmias, enzyme release and myocardial necrosis in isolated rat hearts with coronary occlusion and reperfusion. Naunyn Schmiedebergs Archives of Pharmacology,

1988; 338: 88-95).

When tested at a concentration of 5% and 10%, Karal® produced no significant effect in the duration and incidence of reperfusion arrhythmias. However, a significant reduction of post-ischemic arrhythmias was observed when Karal® 20% was applied to the perfusion solution (**Figure 1A**). The antiarrhythmogenic effect of Karal® 20% was evidenced by a decrease of approximately 90% in ASI (1.2 ± 0.489 versus 12.0 ± 0.003 for the control group). In addition, the occurrence of irreversible arrhythmias was abolished in the hearts perfused KRS containing Karal® at the highest concentration tested (**Figure 1B**). In control hearts, ventricular tachycardia and/or fibrillation during reperfusion were observed in 100%, whereas Karal® 20% inhibited the generation of these arrhythmias in 40% of the hearts.

Using Ang. (1-7) alone ASI was reduced significant to 5.60 ± 1.93 ($p = 0.01$) compared to control, while the combination of Ang. (1-7) and Karal® 5% ASI showed a highly significant reduction of ASI to 4.67 ± 0.66 ($p = 0.0001$; **Figure 1C**). In addition, the occurrence of irreversible arrhythmias was abolished in the hearts perfused KRS containing the combination of Ang. (1-7) with Karal® 5% (**Figure 1D**).

Karal® 20% induced an immediate and significant increase of the coronary flow during basal time, occlusion as well as reperfusion, whereas Karal® 5% induced only a slight but significant raise of the coronary flow (**Figure 2A and B**). However, no changes in the flow were observed in the Karal® 10% group.

The groups receiving Karal® alone or in combination with Ang(1-7) 0.22 nmol/L presented an increase of the coronary flow compared to the control group or that with Ang (1-7) alone. The increase was significantly higher using the combination Karal® 5% + Ang (1-7) 0.22 nmol/L compared to Karal® 5% alone (**Figures 2C and 2D**).

Karal® 20% enhanced the systolic tension significantly throughout the entire experiment. In contrast, Karal® 5% and 10% did not improve the contractile function during and after occlusion (**Figure 3A**).

The diastolic function was preserved throughout the whole experiment using the 20% Karal® concentration. On the opposite, Karal® 5% and 10% increased the diastolic tension during reperfusion (**Figure 3B**).

The combination Karal® 5%+ Ang (1-7) 0.22 nmol/L showed a significant increase in the systolic tension (**Figure 3C**), the diastolic tension however was not significantly different within all groups (**Figure 3D**).

EXAMPLE 2 - Nitrite Measurement

To determinate if Karal® or its ingredients affect in any way on nitrite or NO, five perfusate samples were collected in the basal period (minute 15), occlusion period (minute 5 and 15) and reperfusion period (minute 5 and 25). The samples were immediately stored at -80°C for further nitrite measurement, which was determined by measuring the 2, 3-Diaminonaphthalene (DAN) assay in perfusate as described by Misko (Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. *Analytical Biochemistry*, 1993; 214(1):11-6). Nitrite concentrations during basal, occlusion and reperfusion are significantly higher using 10 and 20% Karal compared to control (Krebs Ringer Solution) due to the effect that KARAL was able to convert peroxynitrite to nitrite. Peroxynitrite consumption through α KG, one of the main ingredients of Karal®, led to the formation of succinic acid and nitrite (NO_2^-). In the heart, NO_2^- has the potential to act as an endogenous store of NO especially liberated during ischemia, when the generation of NO from L-arginine by NOS enzymes is impaired.

EXAMPLE 3 - Determination of peroxynitrite (ONOO-) scavenging function of Karal® and Ang (1-7) with chemiluminescence technique

ONOO- was prepared according to Hughes and Nicklin (Hughes MN, Nicklin HG. The chemistry of pernitrites. Part I. Kinetics of decomposition of pernitrous acid. *Journal of the Chemical Society*, 1968, 450-452). The ONOO- concentration was determined spectrophotometrically at 302nm with a coefficient of extinction of 1670 M⁻¹cm⁻¹. Consumption of peroxynitrite was measured with luminescence technique according to Radi et al (Radi R, Consgrove TP, Beckman JS, Freeman BA. Peroxynitrite- induced luminol chemiluminescence. *Biochemical Journal*, 1993; 290:51-57). 5 μ L of 30mM ONOO- was transferred into a

white 96 well microtitration plate (Nunc, Denmark). 0, 5, 7.5, 10, 15, 20 and 25% of the Karal® solution was pipetted directly to ONOO⁻. Immediately after one second luminol was added (total reaction volume 200µL) and the chemiluminescence signal was detected on a BMG luminescence plate reader (Lumistar, BMG, Germany) each second between 0 and 40 seconds. Luminescence signal is expressed with counts per second (cps).

Figure 5 shows that Karal® was able to decrease the oxidative damage in a dose dependent manner: 5% of Karal® decreased the signal to 58% compared to the control signal without, 7.5% to 16%, 10% to 11%, 15% to 3%, 20% to 1% and 25% nearly to 0%

Interaction studies of ONOO⁻ with Ang. (1-7) were measured with the same procedure using different concentration of Ang.(1-7) (0 – 0.16 mM) which was dissolved in 10 mM PBS pH 7.4.

Figure 6 shows that Ang. (1-7) was able to react with ONOO⁻ at very low concentration. The chemiluminescence signal of ONOO⁻ with 0.8 mM Ang. (1-7) was reduced to nearly 50% compared to control signal ($p < 0.001$). Interestingly with lower Ang. (1-7) concentrations the inhibition of chemiluminescence signal increased: 63.4% at a concentration of 0.16 mM Ang. (1-7) and at least to 75% at 0.04 mM Ang. (1-7) compared to control ($p < 0.001$).

Chemiluminescence results show that Ang. (1-7), containing both amino acids L-tyrosine and L-arginine, reacts easily with ONOO⁻. The synergistic effects of Karal® prevent the nitration of Ang. (1-7) and therefore prolong the function of Ang. (1-7) leading to a significant increase of coronary flow and systolic tension and a significant decrease of diastolic tension during warm and cold ischemia.

EXAMPLE 4 – Preparation and perfusion of isolated hearts (Cold Ischemia)

Laparotomy was performed in rats prior anaesthetized with 0,1 ml /100 mg Thiopental sodium. The inferior vena cava (IVC) was exposed and 400 IU of heparin were administered intravenous, using an insulin syringe. After one minute the IVC and abdominal aorta (AAo) were cut for exsanguination.

Thoracotomy was performed rapidly, the thymus removed gently and the superior vena cava (SVC) cut 1-2 mm before its confluence into the right atrium. Warm blood was removed from the thoracic cavity by using 1-2 pieces of gauze. The brachiocephalic trunk was clamped 2-3 mm after its outlet from the aortic arch using a curved haemostat. A second haemostat was attached between the outlet of the brachiocephalic trunk and the left carotid artery.

Cardioplegic solution (Celsior®, IMSTIX SANGSTADT, France) was applied via the brachiocephalic trunk using a subcutaneous needle attached to a 5ml syringe. The tip of the needle had been bent before, forming a 90° angle. Cardioplegia was performed air-free and under constant pressure (5mL /min) to avoid volume overload. Simultaneously the heart was cooled from the outside using cardioplegic solution and ice. After cardiac arrest the hearts were harvested rapidly by cutting off the AAO, the IVC 1-2 mm before its confluence with the RA, and both lungs.

The hearts were either stored in (1) Celsior® solution (n=10), or in Celsior® solution containing (2) 20% of Karal® (n= 6), (3) (0.22 nmol/L) Ang. (1-7) (n=7) or (4) the combination of both (n=4), at 4°C for 6 hours before being attached to the Langendorff perfusion system described prior.

After a short period of stabilization (10 minutes, KRS perfusion), the hearts were perfused for 60 minutes with (1) KRS (control, n=10), (2) KRS + Karal® 20% (Karal®, n= 8), (3) KRS + Ang-(1-7) (0.22 nmol/L) (Angiotensin, n=8) and (4) KRS + Ang-(1-7) (0.22 nmol/L) + Karal® 20% (Ang + Karal®, n= 5). The first 10 minutes of stabilization were not used for statistical analysis.

Immediately after reperfusion the left ventricle was harvested and fixated for histology and scanning electron microscopy.

Coronary flow was enhanced by Karal 20% alone (* $p < 0,001$ vs. control and ° $p < 0,001$ vs. Ang (1-7)) and the combination of Karal 20% and Ang. (1-7) significantly (# $p < 0,003$ vs. Karal® + Ang (1-7), **Figure 7**).

Systolic tension was increased using the combination of Karal 20% and Ang. (1-7) significantly (* $p < 0,001$ vs. control, **Figure 8**).

EXAMPLE 5 – Histological analysis

For histological examination, tissue blocks of the left ventricle were fixated in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L cacodylate buffer of pH 7.2 were embedded in historesin (LKB, Bromma, Sweden) and cut to obtain 4 μ m sections, which were stained with a mixture of methylene blue-azure II and basic fuchsin (Humphrey CD, Pittman FE. A simple methylene blue-azure II basic fuchsin stain for epoxi- embedded tissue sections. Stain Technology, 1974; 42:9-14).

For ultrastructural examination rat hearts were fixated in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde, post-fixated in 2 % OsO₄, dehydrated, critical point dried and gold sputtered. Representative electron micrographs were captured using a DSM 950 (Zeiss). All the groups were compared to a native, freshly excised rat heart.

At the ultrastructural level the morphology of endothelial cells of freshly excised and immediately fixed hearts (**Figure 9A**) closely resembled to endothelial cells of hearts perfused with Karal solution + Ang. (1-7) (**Figure 9B**). The endothelial cells of both groups showed a polygonal shaped morphology with numerous microvilli at the cell surface and clearly visible contact zones between the cells. The endothelial cells of hearts perfused with Kreb's solution exhibited a smoother cell surface with only few microvilli (**Figure 9E**).

Histological examination of the left ventricle exposed to Karal® reperfusion and Ang. (1-7) showed intact endothelium and underlying connective tissue of the endocardium. Also the heart muscle cells had a normal appearance (**Figure 10B**). The cardiac wall exposed to Kreb's reperfusion (**Figure 10E**) contained besides areas with normal morphology, also regions with structural alterations, especially in areas of the endocard and subendocardial heart muscle cells. In addition, the connective tissue underlying the endothelial sheet showed degenerating processes reflected by gaps. Some cardiomyocytes showed varying degrees of disintegration up to myocytolysis reflected by vacuolization.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Statistical analysis was performed by the one-way ANOVA followed by Newman Keul's test or by two-way ANOVA followed by the Bonferroni test. $p < 0.05$ was considered to be significant.

CLAIMS

1. An antioxidant composition, comprising a combination of
 - at least one carbonic acid selected from the group of mono- and polycarbonic acids,
 - at least one mono- or polysaccharides derivatized heterolytic aldehyde,
 - at least one selenium-compound such as seleniummethionine, seleniumcysteine, or selenite,
 - at least one compound selected from the group of sulfur-, disulfide- or sulfhydryl-containing amino acids such as N-acetyl-L-methionine and N-acetyl-L-cysteine, and aminosulfonic acids such as taurine,
 - at least one carbohydrate, and
 - at least one Mas receptor agonist such as angiotensines.
2. The antioxidant composition according to claim 1, wherein the carbonic acid is selected from the group of α -ketoglutarate, iso-citrate, or a pharmaceutically acceptable salt thereof.
3. The antioxidant composition according to claim 1 or 2, wherein the mono- or polysaccharides derivatized heterolytic aldehyde is selected from the group of 5-hydroxymethylfurfural, dehydroascorbic acid, maltol, vanillin and furan-2-aldehyde.
4. The antioxidant composition according to any one of claims 1 to 3, wherein the Mas receptor agonist is selected from the group consisting of angiotensines such as angiotensin (1-7) and angiotensin (1-7) analogues.
5. The antioxidant composition according to any one of claims 1 to 4, wherein the carbohydrate is selected from the group consisting of monosaccharides, disaccharides, oligosaccharides and polysaccharides.
6. The antioxidant composition according to any one of claims 1 to 5, comprising a combination of α -ketoglutarate, 5-hydroxymethylfurfural, N-acetyl-selenium-L-methionine, N-acetyl-L-methionine, glucose and angiotensin (1-7).

7. The antioxidant composition according to any one of claims 1 to 6, comprising a combination of
- 0.01 to 500 g/L α -ketoglutarate,
- 0.001 to 100 g/L 5-hydroxy-methylfurfural,
- 1.4 to 2.3 mg/L N-acetyl-selenium-L-methionine,
- 70 to 230 mg/L N-acetyl-L-methionine,
- 0.1 to 800 g/L mM glucose, and
- 0.22 ng/L to 2.2 mg/L angiotensin (1-7).
8. A pharmaceutical composition comprising the antioxidant composition according to any one of claims 1 to 7 and a pharmaceutically acceptable carrier.
9. The antioxidant composition according to any one of claims 1 to 7 or the pharmaceutical composition according to claim 8 for treatment of conditions and diseases associated with oxidative and nitro-stress.
10. The antioxidant composition according to any one of claims 1 to 7 or the pharmaceutical composition according to claim 8 for use in a solution for preserving organs and tissues.
11. The antioxidant composition according to any one of claims 1 to 7 or the pharmaceutical composition according to claim 8 for use in the preparation of a perfusion solution for preserving organs and tissues during the process of transplantation.
12. The antioxidant composition according to any one of claims 1 to 7 or the pharmaceutical composition according to claim 8 for treatment of cardiovascular diseases, preferably conditions and diseases associated with ischaemia/reperfusion.
13. A graft treated with an antioxidant composition according to any one of claims 1 to 7 or a pharmaceutical composition according to claim 8.
14. The graft according to claim 13 selected from the group consisting of tissue, isolated cells and an organ, preferably a heart.

15. A method comprising pre-treating a graft before transplantation with an antioxidant composition according to any one of claims 1 to 7 or a pharmaceutical composition according to claim 8.

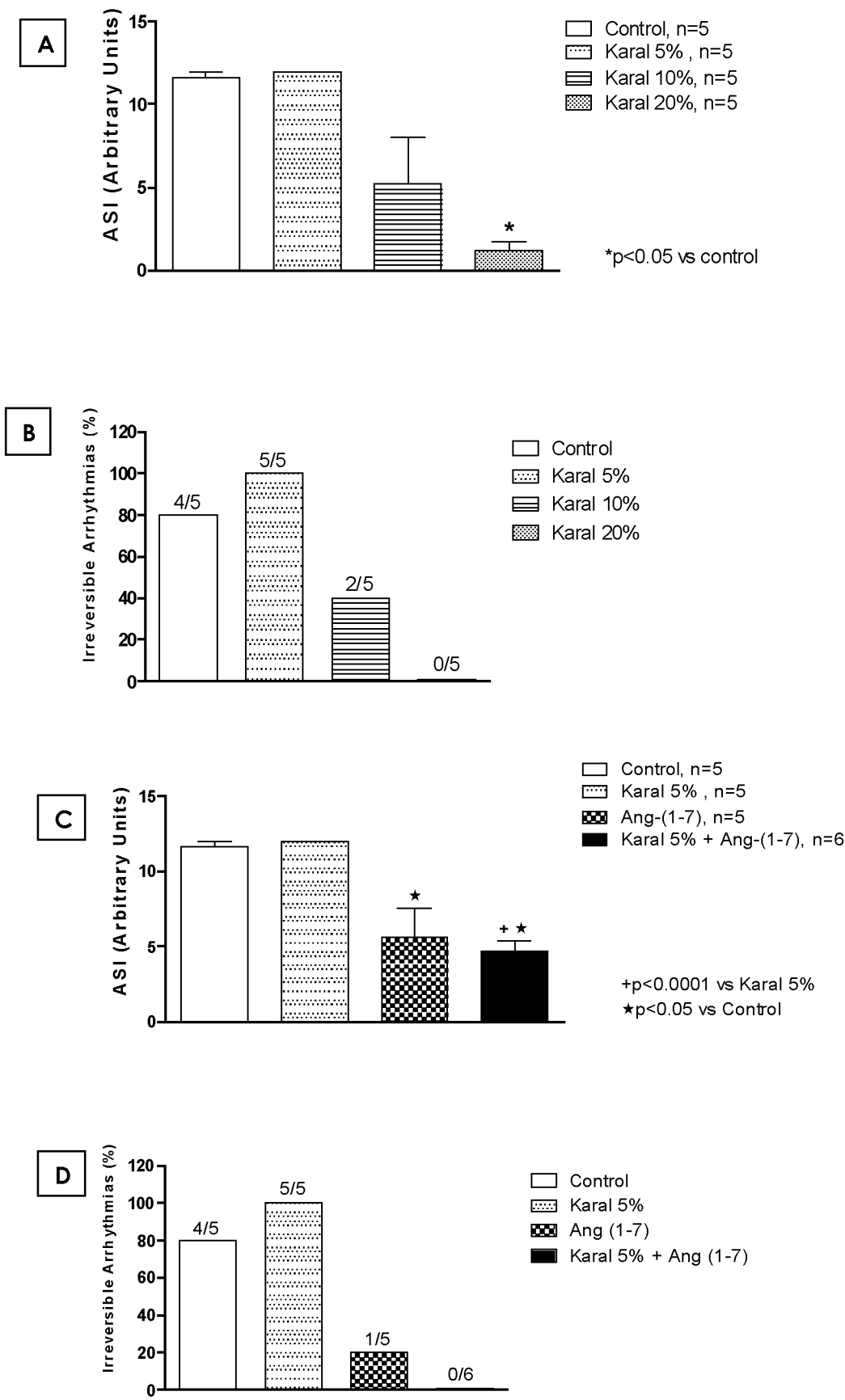


Figure 1

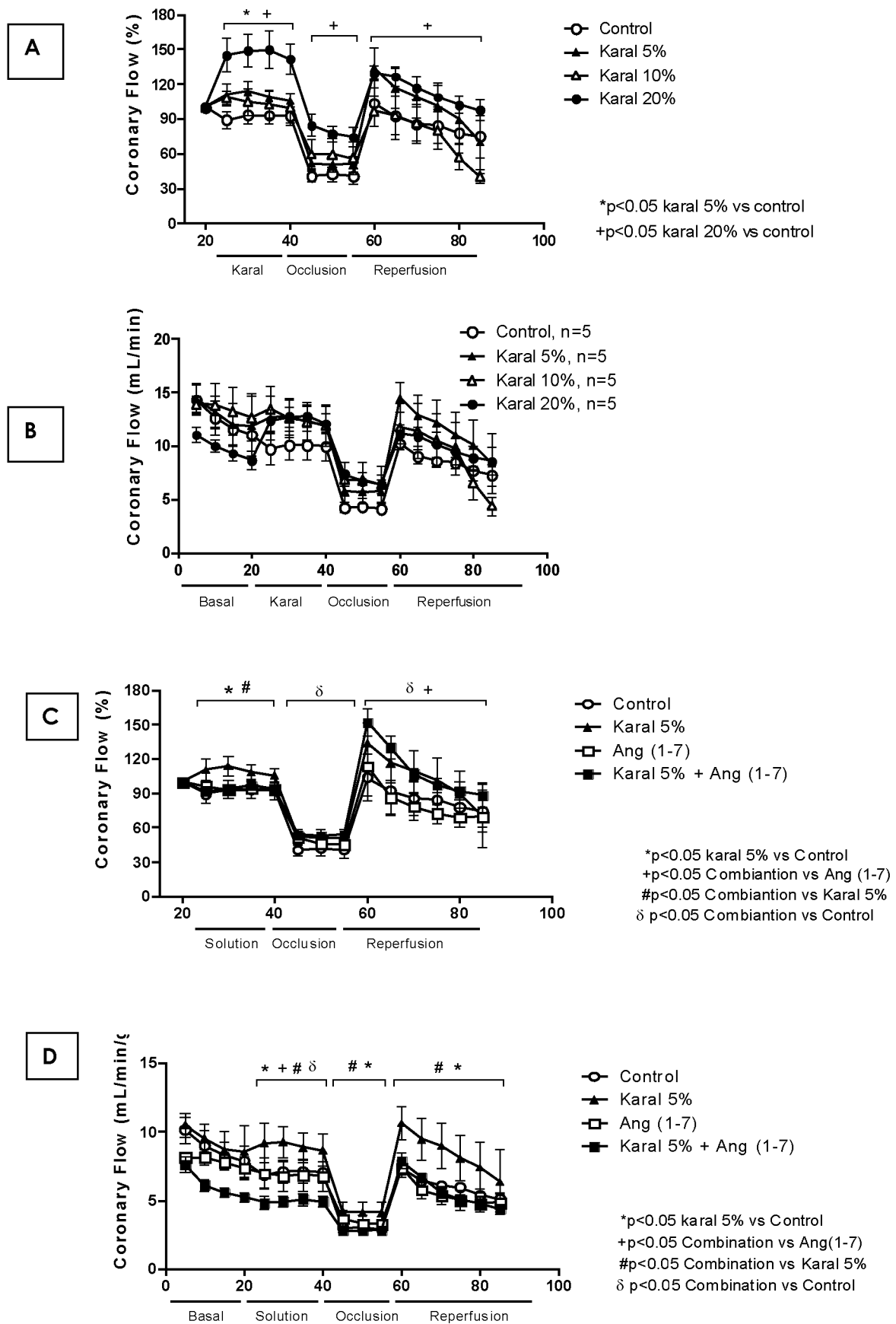


Figure 2

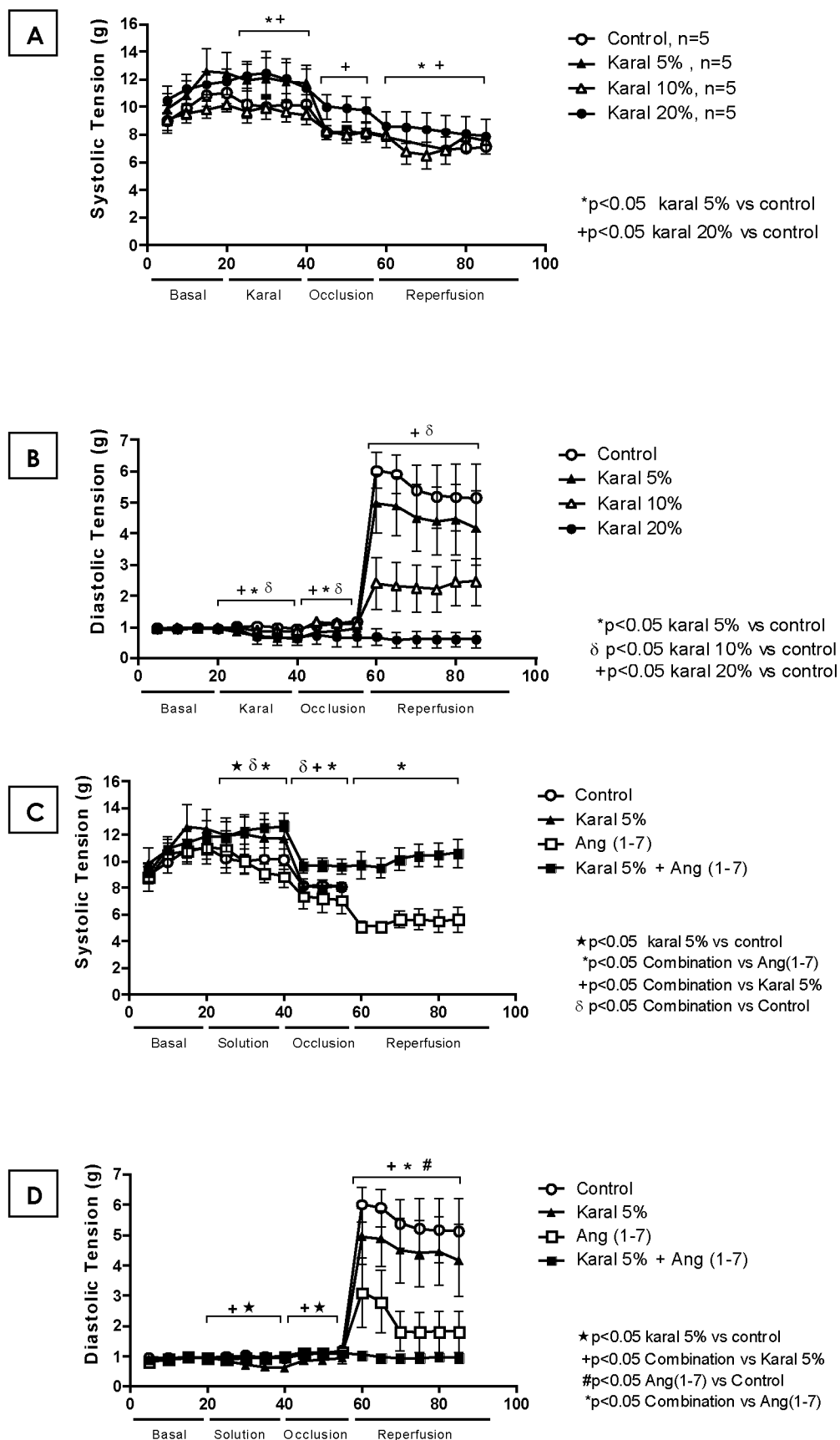


Figure 3

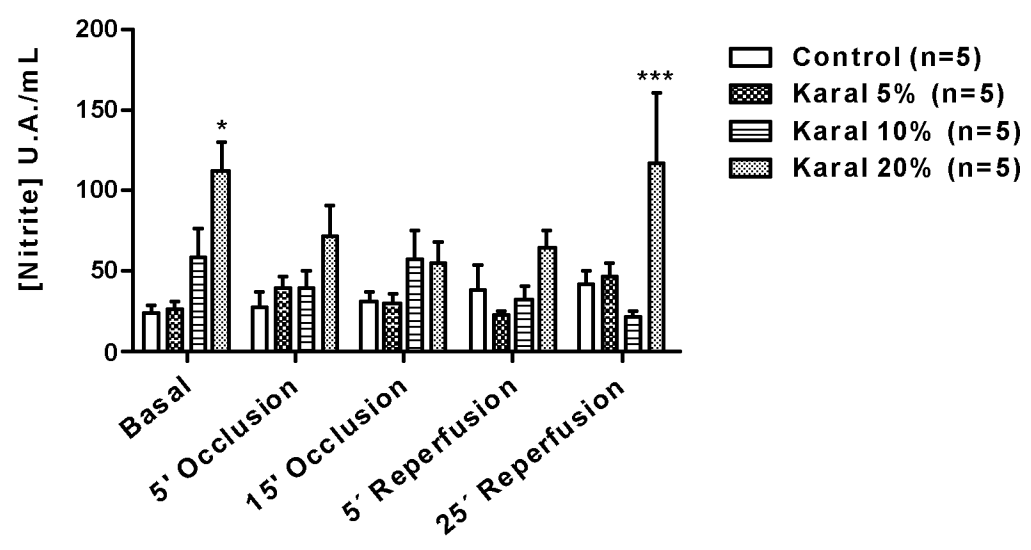


Figure 4

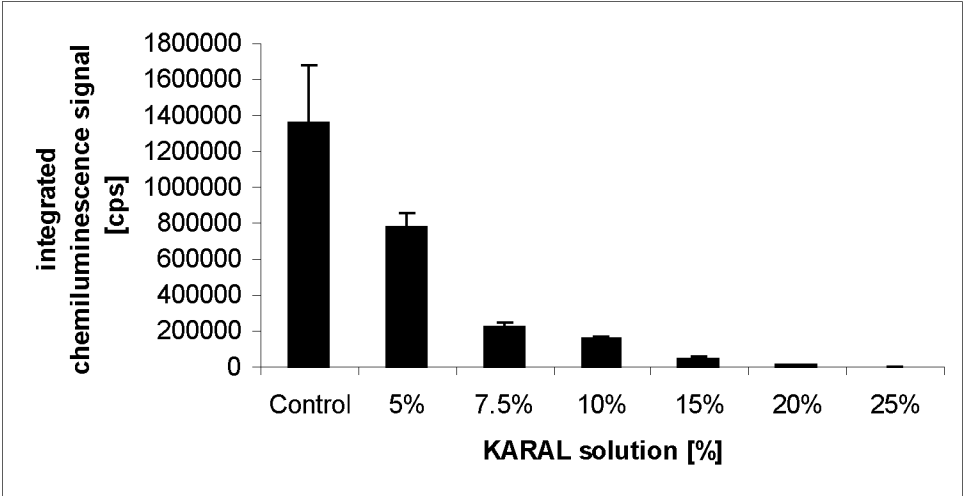


Figure 5

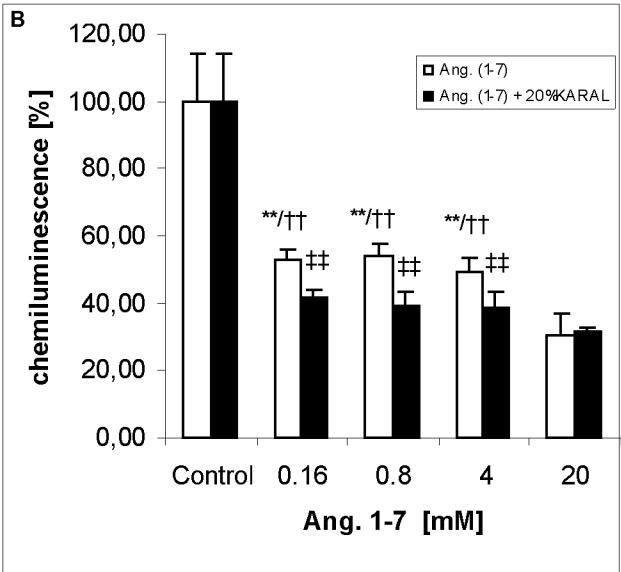


Figure 6

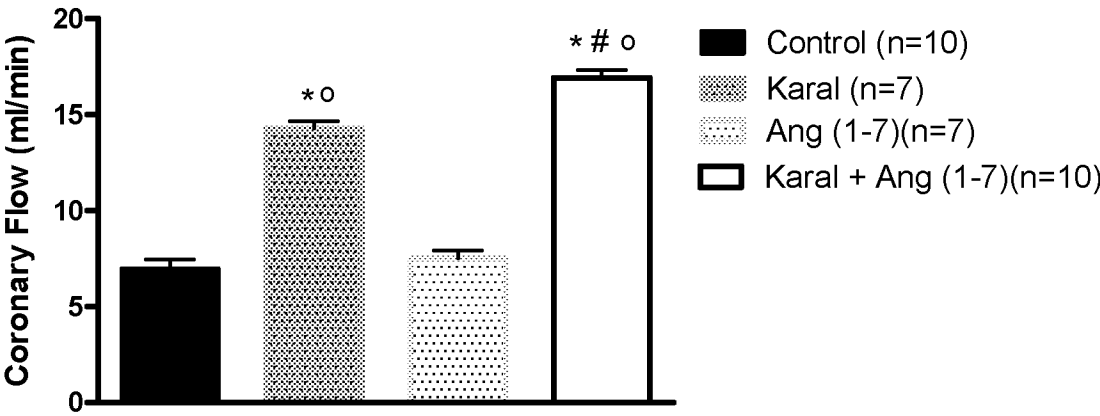


Figure 7

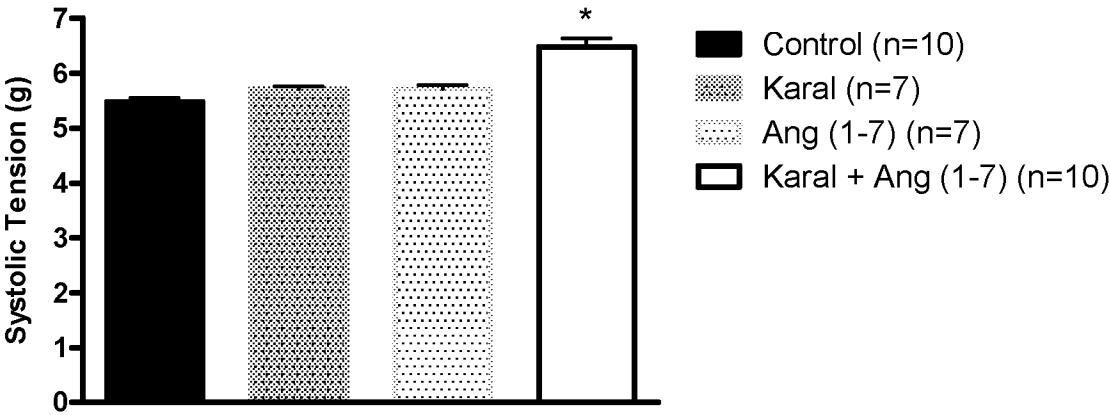


Figure 8

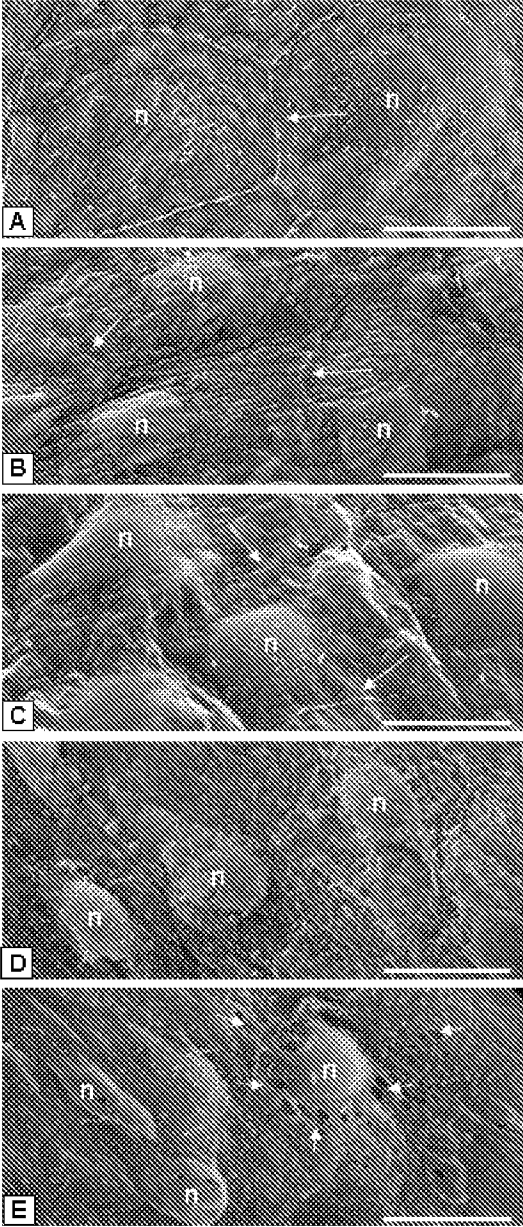
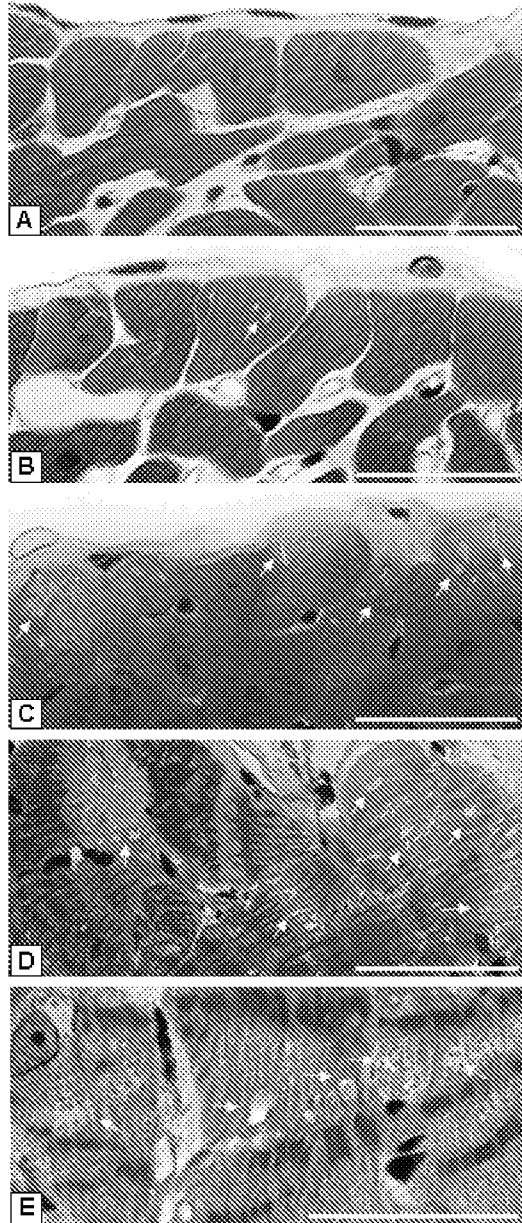


Figure 9

**Figure 10**

INTERNATIONAL SEARCH REPORT

International application No
PCT/AT2012/050049

A. CLASSIFICATION OF SUBJECT MATTER INV. A01N1/02 A61K38/08 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, CHEM ABS Data		
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 3 August 2012		Date of mailing of the international search report 20/08/2012
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Hateley, Martin

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